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Identification of *in vivo* interaction between rabbit hemorrhagic disease virus capsid protein and minor structural protein

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We investigated the ability of the rabbit hemorrhagic disease virus (RHDV) capsid protein (VP60) to interact specifically with the minor structural protein VP10, using an *in vivo* cell-based CheckMate™ Mammalian Two-Hybrid System. RHDV VP60 protein interacted specifically with VP10. Immunofluorescence analysis and co-immunoprecipitation with specific antibodies revealed the existence of biologically important VP60/VP10 complexes. However, when VP60 was divided into two fragments, the interaction between VP60 and VP10 was impaired dramatically. These results will be helpful for further investigating the mechanism of RHDV particle assembly.

rabbit hemorrhagic disease virus, VP60, VP10, protein-protein interaction

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Rabbit hemorrhagic disease virus (RHDV) is an emerging disease in rabbits and is considered the single most economically important disease of rabbits worldwide. The etiological agent has been identified as a *Calicivirus* [1], a positive-sense, single-stranded RNA virus that is antigenically related to European brown hare syndrome virus [2,3]. The complete genome of the virus has been elucidated for the German isolate [4] and shown to comprise a single-stranded, positive-sense RNA genome of 7437 nucleotides. The genome contains two open reading frames (ORFs), the first of which is 2344 codons long (ORF1) and encodes a large polyprotein that contains the viral non-structural proteins, as well as the viral coat protein, at the C terminus. The genome also has a virus-encoded protein, VPg, attached covalently to the 5′ end [5], and is polyadenylated at the 3′ end [6]. In RHDV-infected cells, a 2.2-kb subgenomic mRNA is transcribed that is colinear with the 3′ third of the genomic RNA [7]. This mRNA is thought to represent the major source of the RHDV capsid protein (VP60); however, VP60 is also generated via cleavage of the ORF1-encoded polyprotein [8]. A second ORF (ORF2) is located at the extreme 3′ end of the genome and subgenomic RNAs. The start codon for ORF2 is located at nucleotide 7025, shares a 17-nucleotide overlap with ORF1, but is frame shifted –1 relative to the capsid ORF. RHDV ORF2 is 117 amino acids long and encodes a polypeptide of 12.7 kD (VP10), which is considered to be a component of RHDV virions [9–11]. VP10 is conserved throughout all caliciviruses, suggesting that it may play a role in virus replication or assembly.

To date, the precise mechanism of RHDV virion assembly is not clear. However, recent research has shown that the ORF3 23 kD of Norwalk virus, another important member of the Caliciviridae, is able to interact with VP10 capsid protein and be incorporated into virus-like particles (VIPs)[12]. We assume that the similar interaction also exists in RHDV, and studies on the interactions between VP60 and VP10 will be helpful for exploring the above questions. In this study, we revealed the specific interaction between

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VP60 and VP10 in mammalian cells by the use of different approaches.

1 Materials and methods

1.1 Plasmids construction

The full-length coding regions of RHDV VP60 and VP10 were amplified by RT-PCR using the Moloney murine leukemia virus reverse transcriptase, and *Pfu* DNA polymerase using RHDV genomic RNA isolated from CHA/JX/97 strain of RHDV as a template. The primers, used to amplify VP60, VP10 VP60C, VP60N were listed as following: VP60F: 5′-CTGTCTAGA$_{Xba\ I}$ATGGAGGGCAAAACCCGC-3′; VP60R: 5′-AGTGGTACC$_{Kpn\ I}$TCAGACATAAGAAA-AGCC-3′; VP10F: 5′-CTGTCTAGA$_{Xba\ I}$ATGGCTTTTCT-TATGTCTG-3′; VP10R: 5′-AGTGGTACC$_{Kpn\ I}$TCAAAC-ACTGGACTCGCC-3′. After standard PCR process, PCR products were digested by these enzymes (*Kpn* I and *Xba* I) and cloned into eukaryotic expression plasmids pCI, pBIND or pACT (Promega, USA), respectively. The resulting clones were designated as pCI-VP60, pCI-VP10, pBIND-VP60 and pACT-VP10. To demonstrate that the interaction between VP60 and VP10 depended on the intact structure of VP60, VP60 was divided into two fragments: N-290aa and C-300aa. The corresponding coding regions were amplified by RT-PCR essentially as described above. The primers were as following: VP60CF: 5′-CTGTCTAGA$_{Xba\ I}$ATGC-GGTTTGCCGACATT-3′; VP60CR: 5′-ACTGGTACC$_{Kpn\ I}$TCAGACATAAGAAAAGCC-3′; VP60NF: 5′-CTGTCT-AGA$_{Xba\ I}$ATGGAGGGCAAAACCGGC-3′; VP60NR: 5′-AGTGGTACC$_{Kpn\ I}$TAGAGGGCTTGACCAGCC-3′. PCR fragments were cloned into pBIND. The resulting clones were designated as pBIND-VP60N and pBIND-VP60C. All clones were verified by sequencing, and their sequence information as well as that for all primers is available from the authors upon request.

1.2 Antibodies

Mouse polyclonal serum against RHDV VP10 was used for detection of VP10 protein. Rabbit polyclonal serum against full-length RHDV VP60 was used to detect VP60.

1.3 Cells

HeLa cells were obtained from the China Center for Type Culture Collection and were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C and 5% CO_2.

1.4 Transfection of HeLa cells

HeLa cells were grown to 80% confluence and transfected with different combinant plasmids (Table 1) for 4 h at 37°C with a mixture containing 2–5 μg plasmid DNA and 10 μL Lipofectamine 2000 (Invitrogen, USA), according to the supplier's instructions.

1.5 Luciferase activity assay

HeLa cells at 24 h post-transfection were lysed and luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega).

1.6 Co-immunoprecipitation

pCI-VP60 and pCI-VP10 were co-transfected into HeLa cells. After 48 h incubation, the transfected HeLa cells were lysed in IGEPAL lysis buffer (10 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, 1 mmol/L EDTA, 0.5% IGEPAL CA-630, 1 mmol/L PMSF, and EDTA-free protease inhibitor cocktail (Roche, USA)) for 30 min on ice. Nuclei and nonlysed cells were removed by centrifugation at 1000 × *g* for 10 min. Protein concentration in cleared cell lysates was analyzed using the Bradford assay and the amount of lysate corresponding to 500 μg total protein was incubated with VP10-specific antibody bound to agarose beads for 2 h at 4°C.

The suspension was placed on the protein purification column, agarose beads were washed twice with lysis buffer containing 150 mmol/L NaCl, and proteins bound to sorbent were denatured by boiling in 50 μL Laemmli buffer for 5 min. Ten-microliter aliquots of obtained sample were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. To characterize the interaction between RHDV VP60 and VP10 in HeLa cells, the infected cells were collected at 24 h post-infection by scraping into PBS, followed by centrifugation (10 min, 2000 r/min). Collected cells were treated with 1 mL cold lysis buffer (50 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 10% glycerol, 1 mmol/L DTT, 1 mmol/L PMSF, and EDTA-free protease inhibitor cocktail) for 30 min on ice. Then, cells were sonicated with an ultrasonic homogenizer (20 times for 5 s at 20-s intervals) and the membranous fraction of the cells was collected by centrifugation at 15000 × *g* for 20 min at 4°C. The membrane pellet was treated with 1 mL RIPA buffer (50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, and EDTA-free protease inhibitor cocktail)

Table 1 Groups of plasmid transfection

Groups	pACT	pBIND	pG5luc
Group I: positive	pACT-myOD	pBIND-ID	pG5luc
Group II: negative	pACT	pBIND	pG5luc
Group III: VP10+VP60	pACT-VP60	pBIND-VP10	pG5luc
Group IV: VP10+VP60C	pACT-VP60C	pBIND-VP10	pG5luc
Group V: VP10+VP60N	pACT-VP60N	pBIND-VP10	pG5luc

for 15 min on ice, and sonicated again as described above. The nonsoluble fraction was removed by centrifugation at 15000 × *g* for 20 min. Total protein concentration in the supernatant was analyzed using the Bradford assay and the amount of lysate corresponding to 500 μg total protein was used for co-immunoprecipitation. The VP60 protein was precipitated with specific mouse monoclonal antibody; in reciprocal experiments, VP10 protein was precipitated with the respective rabbit polyclonal serum by use of Protein A Sepharose™ CL-4B (Amersham Biosciences, USA) in RIPA buffer. The immunoprecipitated proteins were denatured by boiling in 50 μL Laemmli buffer for 5 min, and 10-μL samples were used for Western blot analysis with appropriate antibodies, to detect co-immuno-precipitated proteins.

1.7 Cellular localization of RHDV VP60, VP10, VP60N and VP60C protein by immune confocal

The plasmids pCI-VP60, pCI-VP10, pCI-VP60N and pCI-VP60C were transfected into HeLa cells respectively, as described in Section 1.4. The localization of VP60, VP10, VP60N and VP60C was examined using rabbit anti-VP60 and VP10 serum at dilutions from 1∶50 to 1∶400, together with secondary goat anti-rabbit FITC antibody (Sigma, USA). Both the primary and secondary antibody was used in the optimal dilutions for indirect immunofluorescence assay (IFA).

1.8 Confirmation of VP60 and VP10 protein-protein interactions via co-localization using two-color co-immunofluorescence

pCI-VP60, pCI-VP10, pCI-VP60N and pCI-VP60C plasmids were co-transfected into HeLa cells seeded on glass coverslips, in the following two-plasmid combinations: VP60+VP10, VP60N+VP10, and VP60C+VP10. After 48 h, cells were harvested. The relationship of VP60N+VP10, VP60+VP10 and VP60C+VP10 to each other was examined using the mouse anti-VP10 and rabbit anti-VP60 sera at dilutions from 1:50 to 1:400, together with secondary goat anti-rabbit Cy3 antibody (ProteinTech, USA) and goat anti-rabbit FITC antibody (Sigma).

2 Results

2.1 Detection of luciferase activity

Interactions between VP60 and VP10 in transfected cells were studied using the CheckMate™ Mammalian Two-Hybrid System. HeLa cells were grown to 80% confluence and transfected with various combinations of plasmids (Table 1). HeLa cells at 24 h post-transfection were lysed and the activity of luciferase was assayed using the Dual-Luciferase Reporter Assay System. The results showed that the luciferase of Group III (VP10+VP60) was stronger than that of Group II (negative control), suggesting that there was stronger interaction between VP60 and VP10. The results also revealed that the enzymatic activity of Group IV (VP10+VP60C) and Group V (VP10+VP60N) was significantly lower than that of Group III (Figure 1), indicating that the interaction was impaired.

2.2 Subcellular localization of proteins

A high level of transient expression of VP10, VP60, VP60N and VP60C proteins was achieved in transfected HeLa cells. All of the proteins were co-localized predominantly in the perinuclear area (Figure 2(a)–(d)). As a part of VP60, VP60C and VP60N proteins have a low level of transient expression, but they still could be recognized by specific antibodies.

2.3 Confirmation of the interactions between VP60 and VP10 via co-localization using two-color co-immunofluorescence

Co-localization of RHDV VP60 and VP10 was first analyzed by use of co-transfection of HeLa cells with plasmids pCI-VP60, pCI-VPN, pCI-VPC and pCI-VP10. We found that three combinations of co-expressed proteins co-localized in a predominantly perinuclear area (Figure 3(a)–(c)). For VP60 and VP10 co-expression, both proteins were almost exclusively co-localized over the cell cytoplasm (Figure 3(a)). Although co-expression of VP10 and VP60C or VP60N was detected, the amount of complex was very low in cell cytoplasm (Figure 3(b) and (c)). Taken together, these results indicate that co-localization of VP60 with VP10 may depend on the integrity of VP60.

2.4 Interactions of VP10-VP60 proteins

Transient expression of VP10 and VP60 proteins was achieved in transfected HeLa cells and detected by Western blotting (data not shown). Interactions between VP10 and VP60 proteins in transfected cells were studied by co-immunoprecipitation. Protein complexes formed in cells

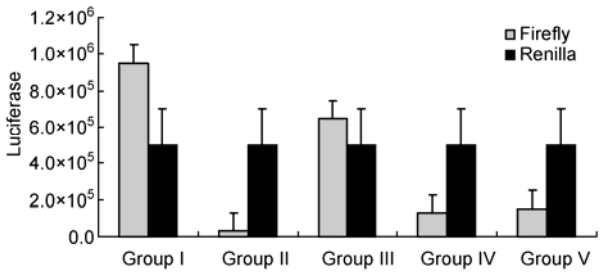

Figure 1 Luciferase detection of Group III (VP10+VP60), Group IV (VP10+VP60C) and Group V (VP10+VP60N) by CheckMate™ Mammalian Two-Hybrid System. For the chart, the firefly luciferase of Group I (positive), Group II (negative), Group III (VP10+VP60), Group IV (VP10+VP60C) and Group V (VP10+VP60N) was detected, which was normalized by Renilla luciferase.

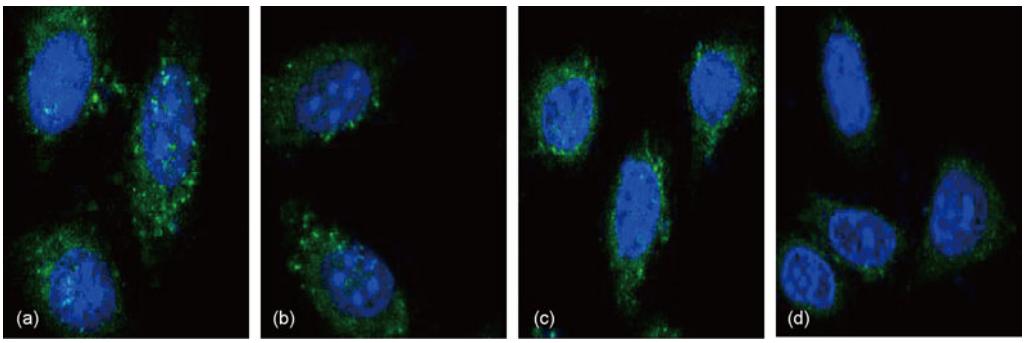

Figure 2 Subcellular localization of VP10, VP60, VP60N and VP60C proteins. HeLa cells were analyzed 48 h post-transfection. The names of the expressed proteins are shown on each panel. Recombinant VP10 (a), VP60 (b), VP60N (c) and VP60C (d) proteins in HeLa cells were detected with rabbit anti-VP60 and VP10 sera at dilutions from 1:50 to 1:400, together with the secondary goat anti-rabbit FITC antibody. Images obtained by confocal Bio-Rad MRC-1024 microscope.

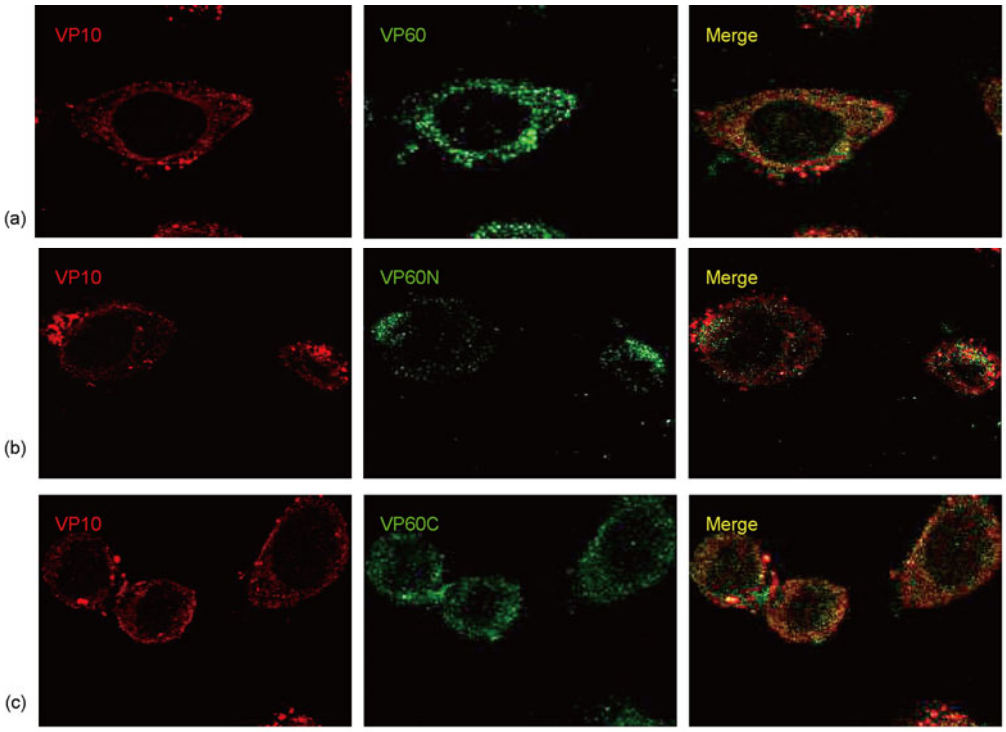

Figure 3 Subcellular localization of VP10 and VP60, VP10 and VP60N, and VP10 and VP60C in HeLa cells 48 h post-transfection. (a) HeLa cells double-transfected with pCI-VP10 and pCI-VP60. Cells were fixed with 4% paraformaldehyde and VP10 was detected by mouse anti-VP10 polyclonal antibody and anti-mouse FITC-conjugated antibody, VP60 was detected by rabbit anti-VP60 polyclonal antibody and goat anti-rabbit Cy3 antibody. (b) HeLa cells double-transfected with pCI-VP10 and pCI-VP60N. Cells were fixed with 4% paraformaldehyde and VP10 was detected by mouse anti-VP10 polyclonal antibody and anti-mouse FITC-conjugated antibody. VP60N was detected by rabbit anti-VP60 polyclonal antibody and goat anti-rabbit Cy3 antibody. (c) HeLa cells double-transfected with pCI-VP10 and pCI-VP60C. Cells were fixed with 4% paraformaldehyde and VP10 was detected by mouse anti-VP10 polyclonal antibody and anti-mouse FITC-conjugated antibody. VP60C was detected by rabbit anti-VP60 polyclonal antibody and goat anti-rabbit Cy3 antibody. Names of proteins are indicated in each panel.

transfected with pCI-VP10 and pCI-VP60 were precipitated under native conditions bound to agarose beads, and co-precipitated proteins were detected with rabbit polyclonal antibodies against VP10 (Figure 4).

3 Discussion

There are only two structural proteins (VP60 and VP10) in mature RHDV virions. VP60 is the major capsid protein and can self assembly without the need for any other protein. However, it has been shown that the expression level of VP60 is related to that of VP10 [13,14]. VP10 is the minor structural protein of RHDV, and its exact biological function is not clear. Some research has suggested that VP10 can increase the stability of the genome and promote capsid assembly, and its expression is regulated by the coding region of VP60. In theory, as a structural protein, VP10

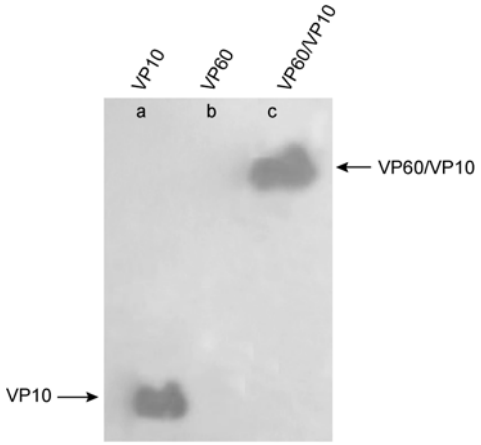

Figure 4 Immunoprecipitation of VP60/VP10 complex from HeLa cells co-transfected with pCI-VP60 and pCI-VP10. Immunoprecipitation was performed by use of agarose beads conjugated with VP10 antibody; precipitated samples were subjected to SDS-PAGE and Western blotting. Twenty micrograms of total cellular protein was loaded in lanes a–c. Lane a: lysate from HeLa cells co-transfected with pCI-VP10. Lane b: lysate from HeLa cells co-transfected with pCI-VP60. Lane c: immune complexes of VP10 and VP60 proteins from HeLa cells co-transfected with pCI-VP60 and pCI-VP10. All the samples were detected by Western blotting using VP10 antibody.

should participate in viral capsid formation. Several investigations on Norwalk virus and *Feline calicivirus* have shown that VP3 (similar to RHDV VP10) can interact with capsid protein and be incorporated into VIPs [15]. However, no experimental data have demonstrated that VP10 may be involved in RHDV particle assembly or that it is a capsid component. We hypothesize that there is a certain interaction between VP60 and VP10, which may promote RHDV capsid assembly.

In the present study, we believe that we proved for the first time that RHDV VP60 and VP10 could form a biologically important complex *in vivo*, and that the complex depends on the intact structure of VP60. If VP60 was divided into two fragments, the interaction could be impaired dramatically. The findings suggest that the interactions between VP10 and VP60 may occur in the process of virion assembly, and VP10 may be involved in this process, which may help with virus maturation. This speculation needs further research for confirmation. In a word, the results of this paper may be helpful for further studies of RHDV virus particle assembly, maturation and release.

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